REVIEW/

RIBOSOME-INACTIVATING PROTEINS FROM PLANTS: PRESENT STATUS AND FUTURE PROSPECTS

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Plant ribosome-inactivating proteins (RIPs) are N-glycosidases which cleave the N-glycosidic bond of adenine in a specific ribosomal RNA sequence. Most commonly RIPs are single-chain proteins (type 1 RIPs), but some (type 2 RIPs) possess a galactose-specific lectin domain that binds to cell surfaces. The latter RIPs are potent toxins, the best known of which is ricin. RIPs have antiviral and abortifacient activities, and, in a widespread application, can also be linked to antibodies or ligands to form immunotoxins or conjugates specifically toxic to a given type of cell.

A number of plant proteins have been identified that catalytically damage eukaryotic ribosomes making them unable to bind the elongation factor 2, and consequently unable to perform the elongation step of protein synthesis[1–3]. These "ribosome-inactivating proteins" (RIPs) are most frequently found as single polypeptide chains (type 1 RIPs). In some cases the RIP is synthesized with a galactose-binding lectin domain. Post-translational cleavage of an intervening sequence results in a protein with two chains linked by hydrophobic bonds and a disulfide bridge. These, termed type 2 RIPs, which can enter cells through the interaction of their lectin moiety with the cell membrane, are among the most potent natural toxins, the best known of which is ricin. As noted by Olsnes and Pihl in their 1982 review[2], ricin has been used since ancient times for medical as well as criminal purposes. In 1978, the Bulgarian playwright and political critic Georgi Markov was assassinated on a London street corner. The assassin, apparently using a modified umbrella, had injected a hollow 2 mm diameter pellet into Markov's leg. Scotland Yard detectives reported the pellet contained ricin and that this was the cause of death[4,5]. That a lethal dose could be delivered in such a small volume is a measure of the potent toxicity of the type 2 RIPs. Type 1 RIPs, which do not possess a lectin domain, do not bind easily to cells, and consequently have a relatively low native cytotoxic activity.

Interest in RIPs is growing due to several recent discoveries. The anti-viral activity of the RIPs has focused attention on their use as potential anti-HIV agents[6–10], and the abortifacient activity of *Tian Hua Fen*, the ancient and widely used Chinese preparation from the root tuber of *Trichosanthes kirilowii*, has been shown to be due to trichosanthin, an RIP present in the root[11–13]. The potent cytotoxicity of cell-targeted RIPs makes them excellent candidates for use in immunotoxin or ligand toxin synthesis[14,15]. Some old, yet fascinating, questions still remain, however. Why do some plants store large amounts of these proteins in their seeds? How can these proteins be synthesized without damaging host protein synthesis? We shall discuss recent advances in the study of these proteins that have revealed much about their chemical and biological properties and that have shed some light on these questions.

DISTRIBUTION

A list of type 1 RIPs, limited to those that have been characterized by purification to homogeneity, is given in Table 1. Many other plants have shown RIP activities that have not been fully characterized[16–20], and the list will increase as new proteins are identified and purified. RIPs with structural similarities and apparently identical function are present in plants belonging to taxonomically unrelated families. Materials from more than 350 plant species were examined in various laboratories[16–20], and translational inhibitory activity was found in more than one-third. Since these studies were aimed at finding sources of large quantities of RIPs rather than performing a systematic study of their distribution, the apparent frequency of these proteins in plant members of certain families may be a consequence of the fact that, having found a high content of RIP in a given plant, several other plants of the same family were examined. In some materials, the amount of RIPs recovered was unusually high: from 100 g of seeds of *Saponaria officinalis*, 600 mg of

saporins were obtained, corresponding to 10% of the total seed protein[21].

RIPs may be present in several parts of the plant, and from some plant materials (*Saponaria officinalis* and *Momordica charantia* seeds, *Dianthus caryophyllus* and *Phytolacca americana* leaves, for example) one can isolate more than one RIP that show very similar sequences and are presumably isoforms of the same protein. Genomic analyses indicate that different genes encode RIPs which differ by only a few amino acids[22].

A complete list of the type 2 RIPs presently known is given in Table 2. Historically, some of these proteins were well known before the discovery of type 1 RIPs: ricin and abrin were known in the last century. Although only the plant parts used for purification are mentioned in the table, modeccin and volkensin are present both in roots and in seeds of *Adenia digitata* and *Adenia volkensii*, respectively. As with type 1 RIPs, slightly different forms of the same toxin have been observed.

STRUCTURE

Type 1 RIPs have many structural similarities, including molecular weights (26–32 kD) and strongly alkaline isoelectric points, sometimes greater than 10 (ref. 23). Many of them show unusual stability. For instance, saporin, unlike the A chains from type 2 RIPs, is resistant to denaturing agents and proteolytic degradation[21]. Most RIPs are glycoproteins, and full length sequences have been reported for several RIPs from protein, cDNA and genomic analyses[24].

The genomic and cDNA sequences of type 1 RIPs indicate that the proteins are synthesized in a preproform[22,25,26], as they possess sequences resembling consensus secretory signal peptides. Ready et al.[27] have observed that pokeweed antiviral protein is stored in the cell wall matrix of leaf mesophyll cells. The signal sequences may be a mechanism for export to the cell wall matrix. A carboxy terminal extension has been reported for saporin[26,28]. This sequence, which includes a glycosylation site, is homologous to a barley lectin sequence that has been found necessary for transport into vacuoles[29]. A C-terminal extended sequence may act as an inactivator of the RIP, as is the case for ricin (see below). The extension would be removed upon transport to a site where the molecule cannot damage the cell's own protein synthesis mechanism. A non-homologous C-terminal extension

TABLE 1 Type 1 ribosome-inactivating proteins (single-chain proteins).

Source		Name	Toxicity to mice LD_{50} (mg/kg)	Inhibitory effect on protein synthesis: Cell-free IC_{50} (nM)*	Inhibitory effect on protein synthesis: HeLa cells IC_{50} (nM)+	Reference
Caryophyllaceae						
Dianthus caryophyllus (carnation)	leaves	Dianthin 30		0.3	18,000	115
		Dianthin 32	3	0.12	14,000	115
Lychnis chalcedonica	seeds	Lychnin	9	0.17	>3,300	116
Saponaria officinalis (Soapwort)	seeds	Saporin 5		0.041		21
		Saporin 6	4	0.037	2,300	21
		Saporin 9	1.7	0.037	5,400	21
Phytolaccaceae						
Phytolacca americana (pokeweed)	leaves	Pokeweed antiviral protein (PAP)		0.24		117
	summer leaves	PAP II		0.25		118
	seeds	PAP-S	2.6	0.037	33,000	119
	roots	PAP-R	1.2	0.05	>3,300	116
	cels	PAP-C	0.95	0.067	3,400	8
Phytolacca dodecandra	leaves	Dodecandrin		0.043		120
Euphorbiaceae						
Gelonium multiflorum	seeds	Gelonin	40	0.4	34,000	121
Hura crepitans (sandbox tree)	latex	*H. crepitans* RIP		0.17	140	21
Manihot palmata	seeds	Mapalmin	>8	0.05	>3,300	116
Cucurbitaceae						
Bryonia dioica (white bryonyl)	leaves	Bryodin-L	>10	0.09	>3,300	116
	roots	Bryodin	12	0.12	2,240	122
Citrullus colocynthis	seeds	Colocin 1	10.7	0.04	>3,300	116
		Colocin 2	12.6	0.13	1,410	116
Luffa cylindrica (sponge gourd)	seeds	Luffin a		0.002		123
		Luffin b				124
Momordica charantia (bitter gourd)	seeds	Momordin	7.4	0.06	32,000	125
		MAP-30		3.3		9
Momordica cochinchinensis	seeds	Momorcochin	24.5	0.12	2,870	126
Trichosanthes kirilowii	roots	Trichosanthin				13
		TAP-29	8	3.7	1,500	10
	seeds	Trichokirin		0.09		127
Poaceae						
Hordeum vulgare (barley)	seeds	Barley RIP		0.83		128
Triticum aestivum (wheat)	germ	Tritin		2.3		134
Zea mais (corn)	seeds	Corn RIP		2.13		128
Asparagaceae						
Asparagus officinalis (asparagus)	seeds	Asparin 1	20	0.27	>3,300	116
		Asparin 2	2	0.15	>3,300	116
Nyctaginaceae						
Mirabilis jalapa	leaves	*Mirabilis* antiviral protein (MAP)	2			56

*A rabbit reticulocyte lysate system. +Concentration causing 50% inhibition.

without a glycosylation sequence is found in the trichosanthin gene[22].

Ricin has been the subject of several structural studies and is the best characterized of the RIPs. Its amino acid[30,31] and nucleotide sequences from both genomic and cDNA[32,33] have been determined. No introns have been observed in the genomic clones (as is also true for genomic clones of type 1 RIPs). cDNA cloning showed the molecule to be synthesized in a prepro- form as a single polypeptide in which a 12 amino acid linker between the two chains is excised to yield the mature protein[33]. This is similar to diphtheria toxin which also is synthesized as a single polypeptide that is proteolytically processed to two chains. By analogy to diphtheria toxin, the toxin enzymic chain is termed the A chain while the lectin chain is termed the B chain.

An endopeptidase capable of removing the intervening 12 amino acid sequence in preproricin has been isolated from castor bean protein bodies, where the toxin is stored[33]. Proricin, without the signal sequence, but with the intervening sequence intact, can bind galactose but is inactive in the enzymic chain assay[34]. Thus, the castor bean cell appears to protect its own protein synthesis apparatus from inactivation by ricin by expressing an inactive molecule which is activated after export to its final destination. There is no C-terminal targeting sequence as has been seen for the type 1 RIPs. Southern hybridization of ricin genomic DNA reveals a multigene family in the castor plant[32] and several different forms of the protein have been isolated[30,31,35–37].

The processed enzymic chain of ricin is 267 amino acids, while the cell-binding chain is shorter by 5 amino acids. The cell-binding chain contains two sites for glycosylation. Glycosylation results in a B chain that migrates more slowly in gel electrophoresis than the A chain, presumably due to more extensive derivatization. Analysis of cDNA indicates heterogeneity in the number of glycosylation sites in the enzymic chain: either one site or two[33]. In fact, at least two enzymic chains of slightly different size can be purified from seed extracts[37]. *E. coli* expression studies indicate that glycosylation is not required for enzymic activity[38–40]. Abrin A chain isoforms have also been cloned from genomic DNA and expressed in *E. coli*[41]. The clones revealed that abrin may also be synthesized in a prepro- form.

While total sequence similarity among RIPs is generally low (often 15–30%), there can be greater homology in regions that correspond to the proposed active site. Interestingly, the toxin of *Shigella dysenteriae* and the Shiga-like toxins produced by *E. coli* have an apparently identical enzymic activity and display homology in the proposed active site[42,43]. Aligned sequences can be seen in Table 3. A three-dimensional structure of crystalline ricin has been proposed[44]. In the crystal structure of ricin the active site region forms a nucleotide-binding cleft of the type seen in retroviral reverse transcriptases and ribonucleases[45]. Collins et al.[46], using structural modeling methods based on sequence and x-ray diffraction data, have concluded that, while the sequence homology is low, the three-dimensional structures of type 1 and type 2 RIP A chains are very similar.

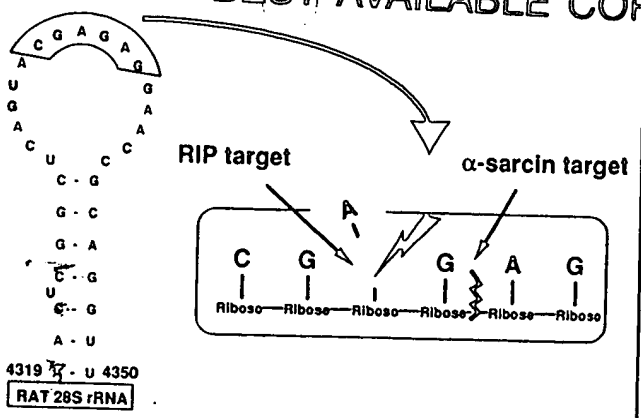

FIGURE 1 The mechanism of action of RIPs. A specific N-glycosidase activity cleaves a single adenine base (A_{4324} in rat) from the ribosomal RNA of the large subunit and causes complete inactivation of the ribosome. The site of attack is adjacent to the α-sarcin site and is contained in an exposed loop termed the α-sarcin domain. The latter toxin cleaves the phosphodiester bond between G_{4325} and A_{4326} in rat 28S rRNA, which also results in loss of ribosome function.

BIOLOGICAL PROPERTIES

All RIPs share the common property of inactivating ribosomes, hence inhibiting protein synthesis. This is due to their highly specific RNA N-glycosidase activity that cleaves the glycosidic bond of adenine$_{4324}$ in rat liver 28S rRNA (Fig. 1). This "Achilles' heel" of the ribosome forms

TABLE 2 Type 2 ribosome-inactivating proteins (two-chain proteins, toxins).

Source		Name	Toxicity to mice LD_{50} (μg/kg)	Inhibitory effect on protein synthesis: Cell-free IC_{50} (nM)*	HeLa Cells IC_{50} (nM)+	Reference
Euphorbiaceae						
Ricinus communis (castor bean plant)	seeds	Ricin (whole)	3.0	84	0.0011	129
		A chain		0.1		129
Fabaceae						
Abrus precatorius (Jequirity)	seeds	Abrin (whole)	0.7	88	0.0037	130
		A chain		0.5		130
Viscaceae						
Viscum album (mistletoe)	leaves	Viscumin (whole)	2.4–80	43	0.008	131
		A chain		3.5		131
Passifloraceae						
Adenia digitata (kilyambiti plant)	roots	Modeccin (whole)	2.4	45	0.0003	132
		A chain		2.3		132
Adenia volkensii	roots	Volkensin (whole)	1.4	84	0.012	133
		A chain		0.37		133

*A rabbit reticulocyte lysate system. +Concentration causing 50% inhibition.

an exposed loop with a stem near the 3'-end of the 28S eukaryotic rRNA and is adjacent to the site of cleavage of rRNA by the *Aspergillus* toxin α-sarcin[47,48]. The slightly faster (less than 1 mm difference) electrophoretic migration of the 28S subunit resulting from the elimination of this single adenine base was first noticed by Endo and his collaborators[49–52], ending a long search for a mechanism of action that could result in the potent inhibition of protein synthesis by RIPs.

The simple removal of one adenine base renders the 60S subunit of eukaryotic ribosomes unable to bind the elongation factor 2 (EF-2), with consequent arrest of protein synthesis. The activity of RIPs is different than that of diptheria toxin and *Pseudomonas aeruginosa* exotoxin[53], which both inhibit protein synthesis at the same step as RIPs by ADP-ribosylating EF-2.

The effected rRNA sequence in and around the loop is highly conserved throughout evolution, being present in ribosomes from bacteria to mammals. The RIPs have the same activity, although at micromolar concentrations, on rRNA purified from bacterial or mammalian sources and on synthetic oligonucleotides[49–51]. Still, their activity varies greatly on intact ribosomes from various organisms. Thus, RIPs are much more active (at nanomolar concentrations) on mammalian ribosomes[49–51], and act to a variable extent on ribosomes from other eukaryotic organisms (plants, protozoan, metazoan)[54,55]. Some RIPs have been reported to be active on *E. coli* ribosomes[56–58]. This has caused difficulty in expressing the *Mirabilis* protein in *E. coli*. Because the assay used in some of these studies is non-quantitative, it is unclear if this will be true for all type 1 RIPs. Ricin A chain, on the other hand, is well expressed in *E. coli*[38–40]. Taken together, these results suggest that each RIP has a specific pattern of activity on target ribosomes of different genera, while ribosomes from each species have a specific spectrum of sensitivity to different RIPs.

The lectin chains of type 2 RIPs bind to sugars with the conformation of D-galactose, and to galactosyl-terminated receptors present on the majority of animal cells. This has two consequences: (1) the cells are agglutinated *in vitro*, and (2) the toxins are internalized. For agglutination to occur, RIPs must be at least bivalent. Indeed, it was demonstrated that the ricin lectin chain has two distinct sites capable of binding galactose, although with different affinities[59–61]. Viscumin may aggregate, thus forming bivalent dimers[62], and the same may well occur with other toxic lectins.

The binding of the lectin chain to the cell membrane allows the toxins to enter the cytoplasm. This is another similarity with diphtheria and many other toxins which also consist of a binding and an active moiety. It has been proposed that the RIP B chains not only bind to cells, but also facilitate the internalization of the enzymic chains through a still unknown mechanism[63,64]. Entry occurs through receptor-mediated endocytosis, and at least ricin reaches the Golgi apparatus[65], where the disulfide bond between the two chains can be reduced by protein-disulfide oxidoreductases[66]. The separated A chains can inactivate ribosomes, thus arresting protein synthesis and killing the cell. Since the process is enzymatic, it is extremely efficient; it is estimated that a single toxin molecule is sufficient to kill a cell[67].

The lectin chains of the various toxins also differ in their cellular interactions, as do the enzymic chains. This is suggested by the different lesions each toxin causes in animals; ricin damaging primarily Kupffer and other macrophagic cells, whereas modeccin and volkensin affect both parenchymal and non-parenchymal liver cells[68–70]. Furthermore, ricin, abrin, modeccin and volkensin are retrogradely transported along axons of peripheral nerves, but only modeccin and volkensin travel along neurons of the central nervous system[71–73]. Ricin injected into the anterior eye chamber, or the submandibular gland or the lip rectus muscle reaches the nervous system[74–76], suggesting that the toxin is taken up by the ends of neurons.

Type 1 RIPs are much less toxic than type 2 because they do not have a lectin subunit, and consequently are less capable of binding to cells and entering them. However, they are highly toxic to some cells, for instance macrophages[77] and trophoblasts[11–13], possibly due to their high pinocytic activity.

The high toxicity of type 1 RIPs to macrophages led to the investigation of their effect on the immune response[78,79]. Indeed, it was observed that administration of type 1 RIPs to mice suppresses the formation of antibodies against T-dependent antigens, provided they are given before the antigen, consistent with the elimination or damaging of macrophages.

The high toxicity to trophoblasts accounts for the abortifacient activity of type 1 RIPs. The roots of the Cucurbitacea *Trichosanthes kirilowii* have been used for more than a thousand years in Chinese traditional medicine to induce abortion. From the roots a protein, trichosanthin, was purified which has abortifacient activity in mice, rabbits, monkeys and humans but not in rats or hamsters[11,12]. Trichosanthin, as now used in Chinese medicine, is injected intramuscularly or directly in the amnion to interrupt normal or ectopic pregnancies with few side effects. It is also used in the therapy of chorioncarcinoma[11,12]. Besides trichosanthin, several other RIPs have been reported to have abortifacient activity. These include momordin, saporin, pokeweed antiviral protein, and, to a lesser extent, gelonin[13]. All type 1 RIPs may have this activity.

The "pokeweed antiviral protein" (PAP) from the leaves of pokeweed, was the first type 1 RIP to be purified, and

TABLE 3 Homologies around the proposed active site between RIPs and shiga-like toxin.

Abbreviations: TCS, trichosanthin; RTA, ricin enzymic (A) chain; SAP: saporin; BRIP, barley ribosome-inactivating protein; SLT, Shiga-like toxin. Asterisks denote residues in all the proteins. Numbers refer to the position of residues in the mature protein. Table taken from Benatti *et al.*[26].

					*	*		*						*		*			*					
TCS	^{169}I	Q	S	T	S	E	A	A	R	Y	K	F	I	.	.	.	L	E	N	S	L	W	L	A	L	S	K	R^{206}
RTA	^{172}I	Q	M	I	S	E	A	A	R	F	Q	Y	I	.	.	.	L	E	N	S	-	W	G	R	L	S	T	A^{217}
SAP	^{171}I	Q	M	T	A	E	A	A	R	F	R	Y	I	.	.	.	E	V	N	-	-	W	K	K	I	S	T	A^{214}
BRIP	^{169}L	L	M	V	N	E	A	T	R	F	Q	T	V	.	.	.	Q	V	N	G	-	W	Q	D	L	S	A	A^{218}
SLT	^{162}V	T	V	T	A	E	A	L	R	F	R	Q	I	.	.	.	T	L	N	-	-	W	G	R	L	S	S	V^{209}

was identified by the antiviral activity of pokeweed leaf extracts on plant viruses. Later it was observed that all RIPs examined prevent replication of viruses both in plants and in animal cells and, conversely, other antiviral proteins were found to be RIPs[16,80–83]. This antiviral activity is attributed to an easier penetration of RIPs through the more permeable membrane of virally-infected cells, with consequent damage to ribosomes, cell death and arrest of viral multiplication[27]. Recently, it was reported that several RIPS inhibit the replication of HIV at concentrations below those affecting cell protein synthesis[6,9,10], suggesting that the effect on HIV replication occurs through a mechanism different from that postulated for the action of RIPs on the replication of other viruses. A formulated preparation of trichosanthin has entered Phase II clinical trials in AIDS patients[7,8]. PAP has been conjugated to antibodies to $CD4^+$ cells with the purpose of targeting the antiviral protein to HIV-infected cells[84]. This targeting causes a thousand-fold increase over free PAP in the inhibition of HIV-1 replication. A thousand-fold higher concentration was also needed for 50% inhibition of protein synthesis of phytohemagglutinin-stimulated uninfected $CD4^+$ cells. Cell-targeting of the anti-viral activity of type 1 RIPs is a promising approach, since a wide variety of viruses are sensitive to these proteins.

CONJUGATES

With the aim of obtaining selectively toxic molecules, many RIPs have been conjugated to carrier molecules capable of delivering them to specific target cell populations. Antibodies, usually monoclonal, are the obvious choice for preparing conjugates ("immunotoxins"), but hormones, growth factors and lectins have also been used as carriers. Detailed studies on targeted RIPs are too many to be reviewed here, and the reader is referred to a recent book[85] and recent reviews on targeted toxins[86] and immunotoxin clinical trials[87].

As experimental tools, ligand toxins and immunotoxins have been used to selectively remove contaminating fibroblasts from cell cultures of pancreatic islets or epithelial cultures isolated from colorectal tumors[88–90]. No doubt this approach could be used to remove other types of cells *in vitro* using selected ligands or antibodies. Immunotoxins have been successfully used for purging *ex vivo* T-cells from bone marrow before transplantation as prophylaxis for graft-versus-host disease[91–93].

In *in vivo* models, very encouraging results using immunotoxins have been obtained in the treatment of transplanted tumors. In patients, on the other hand, positive responses, but few complete remissions, have been obtained. The only published report of a successful therapeutic administration of immunotoxins concerned the treatment of steroid-resistant graft-versus-host disease with an anti-T lymphocyte immunotoxin[94,95]. Other possible medical applications of immunotoxins include the treatment of autoimmune diseases such as thyreopathy and myasthenia gravis[96–98] and the killing of parasites[99–101].

Immunotoxins can also be constructed in an "indirect" manner, by using antibodies against cell antigens, and subsequently adding an immunotoxin prepared with an antibody against the IgG of the same species as the anti-cell antibodies (usually a murine IgG)[102–104]. Another way of delivering toxins to specific target cells is the use of bispecific, hybrid antibodies or F $(ab')_2$ fragments containing one binding site for the target cells and another for the toxin[105,106]. A representation of the various ways of specifically delivering RIPs to target cells is given in Figure 2.

To date, ricin A chain has been the most frequently used RIP to prepare immunotoxins, but more recently modified whole ricin[14] and several type 1 RIPs have been used, namely gelonin, PAP, saporin, momordin, bryodin and barley RIP[15]. Studies with these latter conjugates have demonstrated that type 1 RIPs, when targeted and internalized, make excellent cytotoxic agents[15].

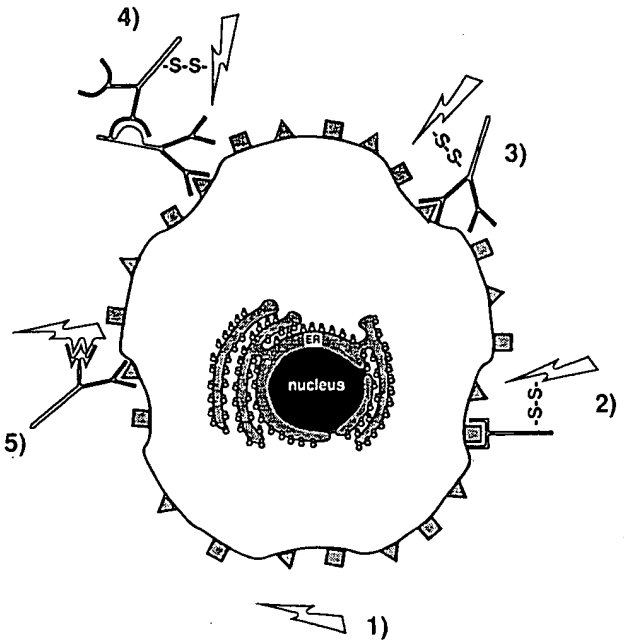

FIGURE 2 Representation of modes of entry of ribosome-inactivating proteins: (1) type 1 RIP (no cell-binding moiety; no mode of entry), (2) type 2 RIP (toxin) with galactose-binding lectin, (3) an immunotoxin with antibody directed to a cell-surface antigen, (4) an "indirect" immunotoxin with antibody directed to an antibody sub-class, a species of which is directed to a cell-surface antigen, and (5) a bispecific immunotoxin in which one antibody binding site is directed to a cell surface antigen and the other directed to a RIP.

PERSPECTIVES

In spite of the considerable information on RIPs gained in recent years, the role of these proteins in nature and their distribution among plants remain enigmatic. The abundance of RIPs in some materials speaks in favor of an evolutionary advantage which would justify their conservation. On the other hand, RIPs seem to be absent or present at very low levels in many plants. They are not produced by some strains of cultured cells from RIP-producing plants, suggesting that they are not essential. This notion, however, is subject to some caution, because in some plant materials RIPs may be present at undetectable levels, could escape detection because they are not active on the ribosomes used to reveal them, could be present in a proform undetectable in the assay or inhibitors of RIPs could be present. Thus RIPs may be ubiquitous in plants, and the possibility that they, or an equivalent, exist in animal tissues should be considered.

Several hypotheses have been formulated about the natural role of RIPs. Possibilities include a role as a defense against viruses or other parasites, or as a means of eliminating altered ribosomes. Further support to the former notion has come from the recent observation that an RIP from barley, acting synergistically with a chitinase and a glucanase, inhibits the growth of fungi[107].

Another feature for further studies will be the mechanism of the different activities of RIPs on ribosomes from

various organisms. It is not clear whether ribosomal protein(s) may interact with RIPs, or may keep the rRNA in the appropriate conformation to be acted upon by RIPs. The different affects on various ribosomes suggest a specificity in the action of RIPs, and it remains to be ascertained whether this is due to the interaction with ribosomal proteins or with rRNA.

Numerous experimental and other applications for RIPs can be envisaged. Both type 1 and 2 RIPs should be useful to study the structure and function of ribosomes. Type 2 RIPs are excellent models to study the entry and fate of proteins into cells, and are becoming useful tools to study the axonal transport of proteins and the interconnections between neurons. Type 1 RIPs are already used to induce abortion, and could be utilized further in veterinary if not in human medicine.

Currently, the most studied application of RIPs is the preparation of antibody and ligand conjugates. These should be exceptional experimental tools for selectively eliminating virtually any given type of cell. Most hopes concern their potential use in the therapy of cancer and other human diseases. With the availability of several, immunologically distinct, toxic moieties and the development of human monoclonal antibodies, immune reactions to conjugates will be overcome. Emerging technologies for the production of antibodies and fragments in bacteria, plant or insect cells should provide a wide variety of powerful carriers. Totally recombinant immunotoxins and ligand toxins have been obtained as chimeric proteins between various cytokines fused to diphtheria and *Pseudomonas* toxin derivatives, and with antibody-toxin chimeras[108–111]. Approaches are also emerging using multistep, pre-targeting strategies to concentrate effectors at the target site while removing unbound, circulating conjugates[112,113].

Conjugates made with RIPs may be among our most potent tools to control the pathologies of hyperproliferation. The last twenty years has enabled technology to catch up with ideas of almost a century ago when Paul Ehrlich suggested using antibody-drug conjugates ("magic bullets") to cure disease[114].

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Maize Ribosome-Inactivating Protein (b-32)

Homologs in Related Species, Effects on Maize Ribosomes, and Modulation of Activity by Pro-Peptide Deletions

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The ribosome-inactivating protein (RIP) from maize (*Zea mays* L.) is unusual in that it is produced in the endosperm as an inactive pro-form, also known as b-32, which can be converted by limited proteolysis to a two-chain active form, $\alpha\beta$ RIP. Immunological analysis of seed extracts from a variety of species related to maize showed that pro/$\alpha\beta$ forms of RIP are not unique to maize but are also found in other members of the Panicoideae, including *Tripsacum* and sorghum. Ribosomes isolated from maize were quite resistant to both purified pro- and $\alpha\beta$ maize RIPs, whereas they were highly susceptible to the RIP from pokeweed. This suggests that the production of an inactive pro-RIP is not a mechanism to protect the plant's own ribosomes from deleterious action of the $\alpha\beta$ RIP. RIP derivatives with various pro-segments removed were expressed at high levels in *Escherichia coli*. Measurement of their activity before and after treatment with subtilisin Carlsberg clearly identified the 25-amino acid intradomain insertion, rather than the N- or C-terminal extensions, as the major element responsible for suppression of enzymatic activity. A RIP with all three processed regions deleted had activity close to that of the native $\alpha\beta$ form.

Many plants produce RIPs, a unique class of proteins that are exceptionally potent inhibitors of eukaryotic protein synthesis (Stirpe et al., 1992; Barbieri et al., 1993). RIPs catalytically inactivate eukaryotic, and in some cases prokaryotic, ribosomes by cleaving the N-glycosyl bond of a single specific adenine residue in the ribosomal RNA (A_{4324} in the case of rat liver ribosomes; Endo et al., 1988). Depending on the species of plant, RIPs can be expressed in leaves, roots, sap, or seeds, often at very high levels. The physiological function of RIPs is at present unclear, although evidence is accumulating that they have a role in plant defense. Leah et al. (1991) have shown that a RIP from barley seeds inhibits the growth of fungal pathogens, particularly when combined with seed chitinases and glucanases. A defensive role against the mechanical transmission of plant viruses has also been proposed (Chen et al., 1991; Bonness et al., 1994). These results have been extended by the observation that transgenic tobacco plants expressing a RIP from barley exhibit increased tolerance to fungal infection (Logemann et al., 1992), and plants expressing a RIP from pokeweed have decreased susceptibility to viral infection (Lodge et al., 1993).

RIPs have been classified into two types (Stirpe et al., 1992): type-1 RIPs are the most prevalent; over 40 have been described. They are typically single-chain, basic polypeptides of 25 to 32 kD with relatively low toxicity to intact cells because they do not readily cross cellular membranes. The rarer type-2 RIPs have arisen from a gene fusion between a type-1 RIP domain and a lectin-like domain. The lectin domain (or B chain) can bind to cell surfaces and mediate the delivery of the RIP (or A chain) into the cytosol of the cell. The RIP A chain can then rapidly and irreversibly inactivate ribosomes to ultimately kill the cell. Thus, most type-2 RIPs described to date are potent cytotoxins, the best known example of which is ricin from castor bean seeds. However, two type-2 RIPs have recently been described that are not cytotoxic, although they have a RIP/lectin structure (Girbes et al., 1993b, 1993c).

We have previously described the purification, characterization, and molecular cloning of a unique type-1 RIP from the endosperm of maize (*Zea mays* L.) (Walsh et al., 1991). Unlike other RIPs, maize RIP accumulates in the seed as an inactive 34-kD precursor (pro-RIP), which is converted into an active form by proteolytic processing. This involves the removal of 16 amino acids (1763 D) from the N terminus, several amino acids from the C terminus, and, surprisingly, 25 amino acids (2708 D) from the center of the RIP polypeptide. The result of this unique series of proteolytic processing steps is a two-chain, activated form of the RIP that we have called $\alpha\beta$ RIP. The two chains are tightly associated but are not covalently linked. Activation of pro-RIP occurs during germination by the action of endogenous proteinases, but can also be performed in vitro by a variety of nonspecific proteinases such as papain and subtilisin Carlsberg. The pro-RIP that we identified by purifying RIP activity from maize endosperm and characterizing the protein responsible for the activity and then isolating its precursor form proved to be homologous to b-32, the Opaque-2-regulated polypeptide of previously

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Abbreviations: IC_{50}, concentration resulting in 50% inhibition; RIP, ribosome-inactivating protein.

unknown function (DiFonzo et al., 1986, 1988; Lohmer et al., 1991). Bass et al. (1992) subsequently reported low levels of RIP activity associated with preparations of b-32. However, their study did not clearly identify and differentiate between inactive precursor forms (pro-RIP or b-32) and proteolytically activated forms of the RIP ($\alpha\beta$ RIP).

Intrigued by the unique maize pro-form of this widespread class of proteins, we asked the following questions regarding maize RIP biology:

1. Do analogous pro-RIP/$\alpha\beta$ forms of RIP exist in relatives of maize, or is the pro-RIP unique to *Z. mays*?
2. Is the pro-form of maize RIP a mechanism to protect maize ribosomes from the activity of the RIP, i.e. are maize ribosomes susceptible to activated maize RIP?
3. Which specific propeptide segment of the pro-RIP is the molecular "switch" that inactivates this otherwise potent enzyme; the N-terminal segment, as in many other zymogenic forms of enzymes, or the intradomain insertion?

In this work, we have determined that pro- and $\alpha\beta$ forms of RIP are not unique to maize but are found in other members of the Panicoideae. We have assessed the activity of both maize pro-RIP and fully activated $\alpha\beta$ RIP on ribosomes from maize and other species to establish what role the precursor may play in suppressing RIP activity in vivo. In addition, we have shown that maize pro-RIP can be expressed at high levels in a soluble form in *Escherichia coli*, and through a series of genetic deletions we have identified the peptide segments of the pro-RIP that are responsible for suppressing enzymatic activity in the precursor. These observations are discussed in terms of the in vivo function of RIPs.

MATERIALS AND METHODS

Plant Material

Seeds of *Tripsacum dactyloides*, *Zea mays mexicana*, *Zea mays parviglumis*, *Zea luxurians*, *Sorghum bicolor* (KFS-1), *Coix lachryma-jobi*, and *Zea mays* (Pioneer 3110) were a gift from Dr. Neil Cowen (DowElanco, Indianapolis, IN).

Genetic Manipulations

Standard methods of DNA purification, restriction enzyme digestion, agarose gel analysis, DNA fragment isolation, ligation, and transformation were as described by Ausubel et al. (1987) and Sambrook et al. (1989). Enzymes used for genetic manipulations were from Pharmacia LKB Biotechnology (Piscataway, NJ), BRL (Gaithersburg, MD), or New England Biolabs (Beverly, MA). Buffers and protocols used were provided by the manufacturer. All genetic manipulations were done in *Escherichia coli* strain DH5α from BRL.

PCR

A Perkin-Elmer Cetus Thermocycler (Norwalk, CT) was used for PCR amplifications. A typical run consisted of a 1-min denaturation step, a 2-min annealing step, and a 3-min extension step. Temperatures used were 94°, 37° or 50°, and 72°C, respectively. After 25 cycles, the reaction was held at 72°C for 7 min for extension of unfinished products. The reaction conditions for amplification were those recommended by Perkin-Elmer Cetus (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 0.2 mM dNTPs, and 2.5 units of *Taq* DNA polymerase or AmpliTaq). Reactions were performed in four separate tubes, each containing 100 ng of template DNA in 0.1 mL of buffer. Primers were synthesized on an Applied Biosystems PCR Mate or 380A DNA synthesizer and were purified on acrylamide gels. About 50 pmol of each primer were included in each reaction. The sequences of the primers are shown in Figure 1.

Construction of Maize RIP Derivatives

pDE600 and pDE601/pro-RIP

The cDNA encoding maize pro-RIP in pUC19 (Walsh et al., 1991) was engineered for bacterial expression using PCR. Primer RIP-1 (Fig. 1) contains termination codons in all three reading frames to halt translation of any vector-encoded polypeptides, upstream of a Shine-Dalgarno sequence followed by the maize pro-RIP ATG. Primer PIR-4 spans the cDNA/pUC 19 junction. The engineered, amplified product was purified from an agarose gel and ligated into the filled-in *Hin*dIII site of the expression vector pGEMEX-1 (Promega, Madison, WI) to create pDE600. Initial expression experiments indicated that co-expression of pro-RIP with the vector-encoded gene 10 product resulted in insoluble aggregates. Removal of the gene 10 coding region (excision of the 918-bp *Xba*I fragment between the T7 promoter and RIP cDNA) to create plasmid pDE601 eliminated this difficulty. Plasmid pDE601 served as the backbone for all other constructions.

pDE602/RIP-ΔN

The RIP gene contained on pDE602 contains a deletion of the N-terminal leader sequence (residues 1–16 of pro-RIP) resulting in polypeptide RIP-ΔN. It was made by replacing the *Nco*I-*Eco*RI fragment of pDE604 with that of pDE601.

```
5' Primers (RIP)

RIP-1   5' GCTTAATTAATTAAGCTTAAAAGGAGGAAAAAAATTATGGCCGAGATAACCCTAGAGCCGAG 3'

RIP-2   5' GCTTAATTAATTAAGCTTAAAAGGAGGAAAAAAATTATGAAAAGAATAGTGCCAAAGTTCACTG 3

RIP-3   5' ACCGTCACCATGGGCCGCGCCGAAATGACCAGGGCCGTCAACGACCTGGCGAAGAAGAAGAAGG
        CGGCTGACCCACAGGCCGACACGAAGAGC 3'

RIP-8   5' AAGGGTCTGGAGACCGTCACCATG 3'

3' Primers (PIR)

PIR-4   5' TATATAGCATGCCGGCCAGTGAATTCGGCACG 3'

PIR-5   5' GCATTGATCAGGCCTCGTCGTCGTCG 3'

PIR-6   5' ATATATATATGAATTCGCCAGGTCGTTGACGGCCCTGG 3'

PIR-7   5' CGGATCCAGCAGTAGCGGCAGCGGCAGTAG 3'

PIR-9   5' TATATAGGATCCGGCAGTAGTTTGATTCTTAACGAG 3'
```

Figure 1. PCR primers used for engineering maize RIP for bacterial expression.

pDE603/RIP-ΔI

Plasmid pDE603 encodes maize RIP-ΔI with the insertion region deleted (residues 162–186 of pro-RIP). The vector fragment was prepared by cutting pDE601 with *NcoI* (cuts approximately midpoint in the cDNA) and *StuI* (cuts at the 3′ end of the coding region). The large vector fragment was purified for ligation with the PCR-engineered 3′ insert fragment. The insert was generated by using pDE601 as template in an amplification reaction using primers RIP-3 and PIR-5. RIP-3 directs deletion of the insertion region of RIP and backs up to the unique *NcoI* site. The PCR product was cut with *NcoI* and *StuI* and gel purified. Ligation with the pDE601 *NcoI-StuI* vector fragment generated pDE603.

pDE604/RIP-ΔNI

The plasmid pDE604 encodes a maize RIP-ΔNI that is deleted for both the N-terminal leader and insertion sequences (residues 1–16 and 162–186, respectively, of pro-RIP). The vector fragment was prepared by cutting pDE603 with *NotI* (cuts between the T7 promoter and cDNA ATG) and treating the ends with T4 DNA polymerase to eliminate single-strand overhangs at the ends. The DNA was then restricted with *NcoI* and gel purified away from the 5′ coding region of the cDNA. The insert was prepared by PCR amplifying a pDE603 template with the primers RIP-2 and PIR-6. The resulting PCR product was cut with only *NcoI* to generate a 3′ sticky end. The 5′ end of the fragment was left blunt for ligation into the filled-in *NotI* site of pDE603. Ligation of the 5′ truncated PCR product and the vector pDE603, cut with *NotI* and *NcoI*, produced plasmid pDE604.

pDE605/RIP-ΔNIC

Plasmid pDE605 encodes a maize RIP-ΔNIC that has deletions of the N-terminal leader and insertion sequences and a segment from the C terminus (residues 1–16, 162–186, and 295–301, respectively, of pro-RIP). The vector was pDE604 cut with *NcoI* and *StuI* and gel purified from the 3′ coding segment. The insert fragment was a PCR product using pDE603 as template with primers RIP-3 and PIR-7. The amplified fragment was cut with *NcoI*, gel purified, and ligated into pDE603 cut with *NcoI* and *StuI*. The resulting plasmid is pDE605.

pDE606/RIP-ΔNICSN1

Plasmid pDE606 encodes a maize RIP-ΔNICSN1 that is identical to that of pDE605/RIP-ΔNIC except that five additional amino acids are deleted from the carboxyl terminus (residues 1–16, 162–186, and 290–301, respectively, of pro-RIP). The 3′ half of the RIP gene present on the *NcoI-BamHI* fragment of pDE605 was replaced with a PCR-derived fragment using pDE605 as template and primers RIP-8 and PIR-9. The PCR fragment was cut with *NcoI* and *BamHI* and ligated directly into the pDE605 vector to create plasmid pDE606.

RIP Expression in *E. coli*

The expression system used was based on the T7 system described by Studier et al. (1990). The expression strain JM109(DE3) is lysogenic for the T7 RNA polymerase gene under *lac* promoter control. Typically, JM109(DE3) (Promega) was transformed with one of the pDE600 plasmids the day before an expression experiment. The freshly transformed cells were harvested from plates and transferred to Luria broth (5×10^7 cells/mL). The cultures were induced immediately with 1 to 10 mM isopropyl-β-thiogalactoside and incubated with vigorous shaking for 2 to 4 h before harvest by centrifugation. Cell pellets were stored at −20°C.

Cells were disrupted by two freeze-thaw cycles and suspension in 2 volumes of lysis buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 150 mM NaCl, 0.1% Triton X-100 [v/v], 1 mg/mL lysozyme, 100 μg/mL DNase, and 100 μg/mL RNase). The cells were allowed to incubate in lysis buffer for 15 min at 37°C. The extract was centrifuged at 4000*g* for 10 min at room temperature. The supernatant was collected and stored at −20°C prior to purification.

RIP Purification and Characterization

The recombinant polypeptides were purified from the bacterial extracts by precipitating protein at 65% ammonium sulfate and dialyzing the resulting pellet into an appropriate buffer for ion-exchange HPLC. Mono Q chromatography with 20 mM Tris-Cl buffer, pH 7.8, was used for recombinant pro-RIP purification and Mono S chromatography with 10 mM sodium phosphate buffer, pH 7, was used for purification of the remainder of the RIP derivatives, except RIP-ΔN, for which 10 mM sodium phosphate, pH 6, was used. Columns were eluted with a NaCl gradient. Typically, a lysate derived from 1×10^{10} cells yielded 5 to 10 mg of purified recombinant protein. Methods for gel electrophoresis, immunoblot analysis, determinations of protein concentrations, and RIP activity have been described previously (Walsh et al., 1991).

Activation by subtilisin Carlsberg was performed in 0.2 M Tris-Cl, pH 7.8, for 1 h using a RIP:subtilisin ratio of 60:1 (w/w). RIP concentrations were 0.2 to 0.6 mg/mL in the reaction, and the reactions were terminated by the addition of PMSF to 3 mM final concentration.

The molecular mass of the β fragment of native αβ maize RIP was determined by electro-spray ionization MS performed at the Harvard Microchemistry Facility by Dr. William S. Lane. Samples of the β fragment from three different preparations of αβ RIP purified from maize kernels were prepared by reversed-phase HPLC. The N-terminal sequences of the β fragments were confirmed as being the same as those previously reported (Walsh et al., 1991).

Immunoblot Analysis

Seeds of maize (*Z. mays* L.) and maize relatives were ground in a mortar and pestle to a fine powder and extracted for 4 h at 4°C with 4 volumes of 25 mM sodium phosphate buffer, pH 7, containing 150 mM NaCl, 25 μg/mL leupeptin, 25 μg/mL antipain, and 1 mM sodium

EDTA. Extracts were adjusted to 1 mg/mL protein and 5 μL was separated using 17 to 27% SDS-PAGE gels from Integrated Separation Systems (Natick, MA). The gels were electroblotted onto a polyvinylidene fluoride membrane, and the blots were developed using rabbit antisera against the purified α and β fragments of maize RIP (Walsh et al., 1991). Bands were visualized using alkaline phosphatase-conjugated goat antirabbit antibody, nitroblue tetrazolium, and 5-bromo-4-chloro-3-indoyl phosphate following the manufacturer's protocol (Bio-Rad, Richmond, CA).

Depurination Assays

Maize ribosomes were prepared from seedlings 72 h after germination, essentially according to the method of Jackson and Larkins (1976). Depurination assays were performed as described previously by Hartley et al. (1991). This involved incubating isolated ribosomes with and without RIP at 30°C in 25 mM Tris-Cl, pH 7.6, containing 25 mM KCl, 5 mM $MgCl_2$, and 1 mM ATP. The RNA was extracted with phenol/chloroform, and 3-μg aliquots were treated with aniline. Aniline-treated and untreated samples were then run on agarose/formamide gels.

In Vitro Protein Synthesis Assays

In vitro protein synthesis assays using rabbit reticulocyte lysate were performed as described previously (Walsh et al., 1991).

RESULTS

Pro-/αβ RIPs in Other Plant Species

We previously identified a unique zymogen form of type-1 RIP from maize that undergoes extensive proteolytic processing to produce an activated αβ form of RIP (Walsh et al., 1991). However, RIPs characterized from the endosperms of other Gramineae species such as wheat and barley are typical type-1 RIPs with no evidence for unusual precursors or αβ forms (Roberts and Stewart, 1979; Asano et al., 1984). To establish whether related plant species may have RIPs similar to those of maize, extracts of seeds from members of the subfamily Panicoideae, of which maize is a member, were analyzed by immunoblotting using antisera against the α and β fragments of maize RIP (Walsh et al., 1991). The following species and subspecies were tested (in approximate order of relatedness to *Z. mays*: *Z. mays parviglumis* (three accessions), *Z. luxurians*, *Z. mays mexicana*, *T. dactyloides*, *S. bicolor*, and *C. lachryma-jobi*. Figure 2 shows that there was immunoreactivity with all of the extracts except the most distantly related species, *C. lachryma-jobi*. Moreover, the pattern of immunoreactivity observed in the extracts was similar, consisting of a band at 32 to 34 kD corresponding to maize pro-RIP and bands at around 16 and 11 kD corresponding to the α and β fragments of the activated form. Therefore, it appears that seeds of many members of the Panicoideae contain RIPs in a pro-/αβ form, i.e. they contain b-32 homologs. This is also supported by Southern blot analysis of DNA from these species using a maize pro-RIP cDNA as a probe (K. Armstrong and N. Cowen, personal communication).

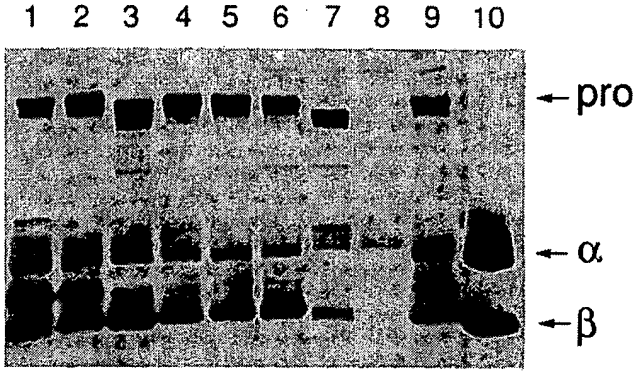

Figure 2. Immunoblot analysis of protein extracts from seeds of *Z. mays* relatives. Each lane contained 5 μg of protein except lane 10, which contained 25 ng of purified maize αβ RIP. Blots were probed with rabbit antisera against purified α and β fragments of maize αβ RIP. The extracts are numbered as follows: lane 1, teosinte (day neutral tunicate); lane 2, teosinte (day neutral); lane 3, *T. dactyloides*; lane 4, *Z. mays mexicana*; lane 5, *Z. mays parviglumis*; lane 6, *Z. luxurians*; lane 7, *S. bicolor* (KFS-1); lane 8, *C. lachryma-jobi*; lane 9, *Z. mays* (Pioneer 3110); and lane 10, purified maize αβRIP.

N-Glycosidase Activity of Maize pro-RIP and αβ RIP

We have shown that there is a significant difference in the RIP activity of maize pro-RIP and αβ RIP in rabbit reticulocyte cell-free protein synthesis assays (Walsh et al., 1991). Other studies with RIPs have shown that many type-1 RIPs not only inactivate heterologous eukaryotic and prokaryotic ribosomes, but also the source plant's own ribosomes (Taylor and Irvin, 1990; Ferreras et al., 1993; Rojo et al., 1993; Bonness et al., 1994). In these cases, the deleterious action of the RIP may be avoided by compartmentalization of the RIP via the secretory system. However, maize RIP is a cytoplasmic, not a secreted protein (DiFonzo et al., 1986). Considering these data, we investigated whether maize ribosomes were susceptible to either form of the RIP by monitoring the effect of pro-RIP and αβ RIP on maize ribosomal RNA. When rRNA is specifically depurinated by the N-glycosidase activity of a RIP, the phosphodiester backbone is rendered susceptible to cleavage by aniline at the site of adenine removal (Endo et al., 1987; Endo and Tsurugi, 1987). This results in the appearance of a small, approximately 300-nucleotide fragment by agarose/formamide gel analysis (the "aniline fragment"), which is diagnostic of RIP action.

Using this type of analysis, we found that maize pro-RIP had no significant effect on isolated maize ribosomes at a concentration of 3.0 μM, corresponding to a pro-RIP:ribosome molar ratio of approximately 8:1. The lack of released aniline fragment is shown in Figure 3, lane 8. These data are in agreement with those of Bass et al. (1992), who tested the susceptibility of maize ribosomes to preparations of b-32. However, these workers did not distinguish between inactive pro- and activated αβ forms of maize RIP. To test the susceptibility of maize ribosomes to activated αβ RIP,

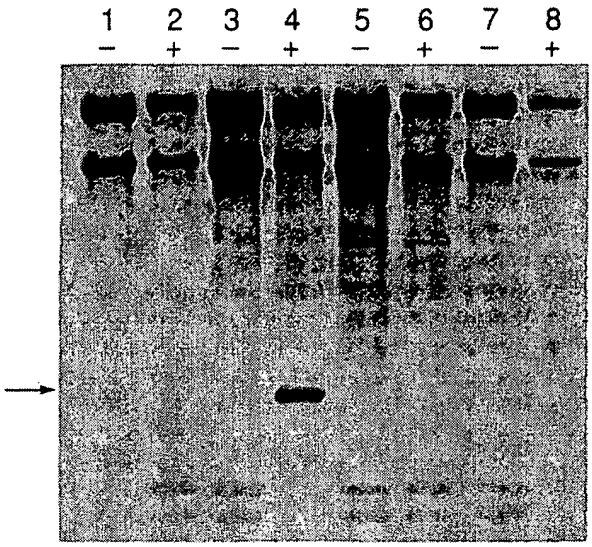

Figure 3. Effect of maize pro-RIP, αβ RIP, and pokeweed antiviral protein on RNA from maize ribosomes. Isolated maize ribosomes (30 μg) were treated with RIP as described in "Materials and Methods." After phenol/chloroform extraction, rRNA was electrophoresed in an agarose/formamide gel and the bands were visualized with ethidium bromide. RNA samples treated with aniline are marked +, those not treated with aniline are marked −. Lanes 1 and 2, Control (no RIP); lanes 3 and 4, 17 nM pokeweed antiviral protein; lanes 5 and 6, 1.8 μM maize αβ RIP; lanes 7 and 8, 3.0 μM maize pro-RIP. The position of the fragment diagnostic for RIP-catalyzed depurination is shown by the arrow.

maize ribosomes were treated with 1.8 μM αβ RIP (αβ RIP:ribosome molar ratio of 4.8:1). Under these conditions, only a very slight trace of the aniline fragment was detected (not visible in the gel shown in Fig. 3, lane 6). Treatment of yeast ribosomes with maize αβ RIP resulted in the release of the aniline fragment, demonstrating that maize RIP is capable of producing an aniline fragment from sensitive ribosomes (data not shown).

In contrast, treatment of maize ribosomes with the RIP from pokeweed (also known as pokeweed antiviral protein) at a 180-fold lower concentration (17 nM; RIP:ribosome molar ratio of 0.044:1) resulted in the release of the aniline fragment, seen in Figure 3, lane 4. Therefore, maize ribosomes are relatively insensitive to both the pro- and αβ forms of maize RIP, but are very sensitive to the action of the heterologous pokeweed RIP (which also has the ability to depurinate pokeweed ribosomes; Taylor and Irvin, 1990; Bonness et al., 1994).

Expression of Maize Pro-RIP in *E. coli*

The T7 expression system described by Studier et al. (1990) was used for expression of maize RIP in *E. coli*. The system relies on the presence of the T7 RNA polymerase for expression of the introduced gene. This positively regulated system allows genetic manipulations to be performed in standard laboratory strains with minimal leaky expression. This was of initial concern because several type-1 RIPs have been reported to have activity against *E. coli* ribosomes (Habuka et al., 1990; Hartley et al., 1991; Girbes et al., 1993a).

Our maize RIP cDNA contained two Met codons near the 5′ end (Walsh et al., 1991). Because we originally isolated the cDNA as a gene fusion from a λ gt11 library, and the N terminus of the naturally occurring pro-RIP was blocked, we had no direct indication as to which Met codon initiated translation. Comparative sequence analysis of the initiator codon context in several maize genes indicated that the 5′ ATG was the more probable start site. This choice was subsequently confirmed by inspection of genomic sequences of b-32 reported by Hartings et al. (1990). The maize pro-RIP cDNA was engineered for expression in *E. coli* using PCR technology. Translation stops in all reading frames immediately upstream of a Shine-Dalgarno sequence were added to the cDNA using appropriate primers. The engineered cDNA was ligated into the expression vector pGEMEX-1 to create plasmid pDE600. When pDE600 was introduced into expression strains containing the T7 RNA polymerase, large amounts of both the vector-encoded gene 10 and maize pro-RIP polypeptides were produced. However, most recombinant material was recovered as insoluble aggregates in the pelleted fraction of bacterial lysates. Removal of the gene 10 coding region from pDE600 to create plasmid pDE601 resulted in both increased production of pro-RIP and in recovery of large amounts of soluble, recombinant protein.

The recombinant pro-RIP was purified to homogeneity and tested for RIP activity in a rabbit reticulocyte lysate protein synthesis assay (Fig. 4). The pro-RIP purified from *E. coli* had a very low level of inhibitory activity on protein synthesis (IC_{50} = 600 nM) relative to native, active αβ RIP (IC_{50} = 0.065 nM). After treatment with subtilisin Carlsberg, the pro-RIP was converted into a potent inhibitor of protein synthesis with an IC_{50} = 0.09 nM, corresponding to

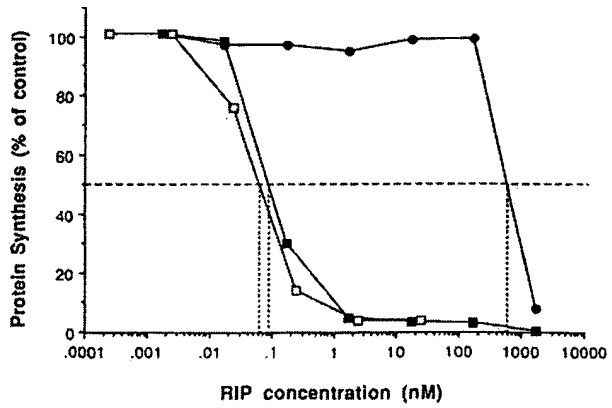

Figure 4. Inhibition of protein synthesis in a rabbit reticulocyte lysate by recombinant maize pro-RIP before and after treatment with subtilisin Carlsberg. The amount of [U-^{14}C]Leu incorporation into protein precipitated by TCA in the presence of varying concentrations of purified, recombinant maize pro-RIP was measured. The recombinant pro-RIP was either untreated (●) or treated with subtilisin (■) as described in "Materials and Methods." The activity of native αβ RIP in the same experiment is also shown for comparison (□). The vertical lines denote the IC_{50} values for each curve.

an approximate 6700-fold increase in activity (Fig. 4). Analysis by SDS-PAGE shows that the recombinant pro-RIP was processed into a two-fragment form that appears very similar to naturally occurring, active $\alpha\beta$ maize RIP (Fig. 5, A and B, lanes 3). These data provide direct evidence that the cDNA we have isolated encodes a polypeptide of low intrinsic RIP activity that can be proteolytically activated to yield a potent RIP.

Determination of Native Pro-RIP C-Terminal Processing

We have previously identified three regions of the inactive pro-RIP that are processed to yield the active $\alpha\beta$ form of maize RIP (Walsh et al., 1991). These consist of a 16-amino acid N-terminal segment, a 25-amino acid insertion in the center of the polypeptide chain, and a segment of unknown length at the C terminus. The extent of C-terminal processing was somewhat ambiguous because of our previous inability to obtain unequivocal sequence data from the C terminus of the β fragment. We have now determined the precise extent of C-terminal processing by an alternative technique. We accurately established the molecular mass of the β fragment of maize $\alpha\beta$ RIP as 11,020 ($\pm$20) D by electro-spray ionization MS (Chait and Kent, 1992) performed on three different samples of β fragment. These were prepared by reversed-phase HPLC from native $\alpha\beta$ RIP purified from maize kernels. Using this value, in combination with the N-terminal sequence of the β fragment and the deduced amino acid sequence of the pro-RIP, the C terminus of the naturally occurring β fragment can be established as Ala^{288}. This gives a predicted molecular mass for the β fragment of 11,074 D, in close agreement with the measured value of 11,020 D. Thus, 14 residues (1,336 D) are processed from the C terminus of maize pro-RIP during activation. The processed regions of maize pro-RIP are therefore residues 1 to 16, 162 to 186, and 289 to 301.

Expression of Modified Maize RIP Derivatives in *E. coli*

Genetic deletions of maize pro-RIP corresponding to each of the naturally processed regions were made and expressed in *E. coli*. This allowed us to investigate the contribution that each of these regions makes in suppressing the activity of maize RIP. The predicted protein sequences for the modified RIP genes are shown in Figure 6. Because of the initial ambiguity regarding the exact C-terminal residue of the β fragment (Walsh et al., 1991), two C-terminal truncation constructions were made. RIP-ΔNIC has seven amino acids deleted from the C terminus of the pro-RIP, resulting in a C terminus that is six amino acids longer than the naturally processed β fragment. The seven deleted residues include all of the charged amino acids naturally processed from the pro-RIP. RIP-ΔNICSN1 is a derivative with five additional C-terminal residues deleted. In both cases, Ala^{292} was changed to a Gly to generate a unique *Bam*HI restriction site. All of the maize RIP derivatives could be expressed as soluble proteins at high levels in *E. coli*. The recombinant products were purified to homogeneity as established by SDS-PAGE analysis shown in Figure 5A. The purified proteins were tested for activity before and after treatment with subtilisin.

Table I shows the IC_{50} values for protein synthesis inhibition in rabbit reticulocyte lysates that were determined for each recombinant RIP derivative. Polypeptides that contain the 25-amino acid insertion (RIP-ΔN, pro-RIP) have low levels of RIP activity, 2000- to 5000-fold lower than $\alpha\beta$ RIP. The level of activity may be even lower, since any slight activation of pro-RIP by minor amounts of contaminating proteinase will result in apparent inhibition of protein synthesis. For example, an IC_{50} value of 600 nM could be accounted for by the presence of about 0.01% of the protein being in the activated form. In contrast to RIP-ΔN and pro-RIP, those recombinant proteins that have the 25-amino acid insertion genetically removed (RIP-ΔNIC, RIP-ΔNICSN1, RIP-ΔNI, RIP-ΔI), are only 2- to 14-fold less active than native $\alpha\beta$ RIP (IC_{50} values of 0.1–1.0 nM). These data clearly identify the 25-amino acid insertion as the primary inactivating element of maize pro-RIP. The presence of the N- and C-terminal segments in proteins that have had the insertion removed (RIP-ΔI, RIP-ΔNI) results in only slightly lower RIP activity (5- to 7-fold) than the fully activated forms, indicating that these segments are not major inactivating elements in the pro-RIP. Although a RIP with only the C-terminal segment deleted was not

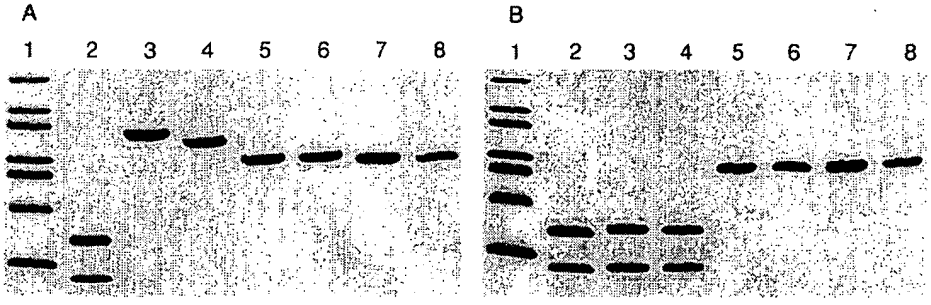

Figure 5. SDS-PAGE of recombinant RIP derivatives before and after treatment with subtilisin Carlsberg. Maize RIP derivatives were expressed in *E. coli* and purified and 0.5 μg were electrophoresed in Phast Gel Homogeneous 20 gels using a Pharmacia PhastSystem. Gels were stained with Coomassie blue. A, Untreated maize RIPs; B, RIPs after treatment with subtilisin Carlsberg as described in "Materials and Methods." Lanes 1, Molecular mass standards (from the top: 66, 45, 36, 29, 24, 20, and 14 kD); lanes 2, maize $\alpha\beta$ RIP purified from maize kernels; lanes 3, recombinant maize pro-RIP; lanes 4, RIP-ΔN; lanes 5, RIP-ΔI; lanes 6, RIP-ΔNI; lanes 7, RIP-ΔNIC; lanes 8, RIP-ΔNICSN1.

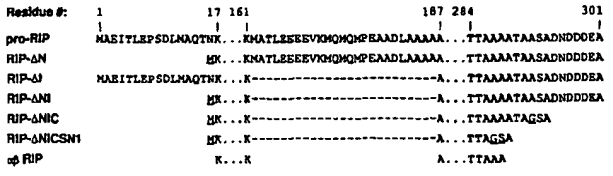

Figure 6. Amino acid sequence differences in maize RIP constructions expressed in *E. coli*. The sequence alignments show the differences between various RIP constructions expressed in *E. coli*. Amino acids that have been changed from the original pro-RIP sequence are underlined. Residue numbers are based on the pro-RIP sequence as previously described (Walsh et al., 1991). Dashed lines denote deleted residues with the presence of a contiguous polypeptide chain. RIP-ΔN has the N-terminal leader deleted, RIP-ΔI has the insertion region deleted, RIP-ΔNI has deletions of both the N-terminal leader and insertion, RIP-ΔNIC has the leader, insertion, and a portion of the C-terminal extension deleted, RIP-ΔNICSN1 is equivalent to RIP-ΔNIC but with an additional C-terminal deletion.

constructed, the relative effect of the C-terminal segment on activity can be inferred by comparing the activities of RIP-ΔI and RIP-ΔNI before and after protease treatment.

The difference in specific activity between the pro-RIP and the most active recombinant form (RIP-ΔNIC) represents a 4,200-fold increase in activity. Those derivatives that have had the insertion deleted represent new forms of maize RIP in which the α and β polypeptides are covalently fused to generate a single polypeptide that retains RIP activity. The specific activity of the most highly modified protein, RIP-ΔNIC, is only marginally less than that of the native αβ form of maize RIP. The potent activity of these fused polypeptides also demonstrates that removal of the insertion is the critical activating factor. Nicking of the polypeptide chain to create separate α and β fragments is not required for activation.

The recombinant RIPs and pro-RIP were treated with the protease subtilisin Carlsberg to investigate proteolytic activation of these forms of maize RIP. In each case processing resulted in higher RIP activity, and the IC_{50} values after protease treatment were approximately equivalent (0.05–0.16 nM; Table I). RIP-ΔNIC activity was unaffected by subtilisin Carlsberg as expected because almost all of the processed regions had been genetically deleted. SDS-PAGE of the RIP derivatives after protease treatment shows that the pro-RIP and RIP-ΔN were processed into two-chain forms of the RIP with α and β polypeptides marginally larger than those of the native form. Cleavage into the two-chain form is a result of proteolytic excision of the internal insertion. Interestingly, polypeptides in which the insertion was deleted genetically and thus were fusions of the α and β fragments (RIP-ΔI, RIP-ΔNI, RIP-ΔNIC) were not susceptible to proteolytic cleavage at the fusion site. Thus, the genetic fusion of the α and β fragments eliminated sensitivity to proteolysis around the internal prosegment region.

DISCUSSION

Maize RIP is the only known RIP that is synthesized as a precursor that undergoes proteolytic processing to a distinctive two-fragment αβ form. This prompted us to survey several related species for proteins with analogous properties. By immunoblot analysis of seed extracts we have found that pro-RIP (or b-32) homologs are not unique to maize but are found in other members of the Panicoideae. The most distant relative of maize that contained cross-reactive pro- and αβ forms of RIP was sorghum. It will be of interest to ascertain whether pro-/αβ RIPs are associated exclusively with Panicoideae or are also found in other plant species. Such a screening project may require alternatives to the conventional technique of monitoring RIP activity in crude extracts, since inactive pro-forms will be overlooked. Sequence analysis of other Panicoideae-type RIPs will provide useful insights into the molecular evolution of both the Panicoideae and RIPs in general.

The question of why these Panicoideae RIPs are expressed as a precursor form is intriguing. Maize RIP is located in the cytoplasm (DiFonzo et al., 1986), in contrast to the more prevalent secreted forms of type-1 RIPs, e.g. trichosanthin (Chow et al., 1990), dianthin (Legname et al., 1991), saporin (Fordham-Skelton et al., 1991), *Mirabilis* antiviral protein (Kataoka et al., 1991), α-momorcharin (Ho et al., 1991), gelonin (Nolan et al., 1993), and pokeweed antiviral protein (Lin et al., 1991). Secretion of these RIPs may provide a mechanism to protect the plant's own ribosomes from the potent deleterious enzymatic action of the RIP. If

Table I. *IC_{50} values for the inhibition of protein synthesis by recombinant maize RIP derivatives before and after treatment with subtilisin Carlsberg*

RIP Derivative	Molecular Mass[a]	Calculated pI[a]	IC_{50}	IC_{50} after Treatment with Subtilisin	Fold Increase in Activity after Treatment with Subtilisin
			nM	*nM*	
proRIP	33,327	5.99	600	0.09	6,700
RIP-ΔN	31,713	7.31	100	0.05	2,000
RIP-ΔI	30,637	8.50	0.98	0.13	7.5
RIP-ΔNI	29,021	9.06	0.69	0.16	4.3
RIP-ΔNIC	28,233	9.50	0.14	0.14	1
RIP-ΔNICSN1	27,848	9.50	0.14	N.D.[b]	N.D.
αβ RIP	27,573	9.50	0.07	0.07	1

[a] Calculated from the deduced amino acid sequence of each derivative. [b] N.D., Not determined.

maize RIP was active against its own ribosomes, then an inactive pro-form would be essential for cytoplasmic accumulation. Previous work examining the activity of maize RIP on maize ribosomes did not distinguish between inactive pro-RIP and activated $\alpha\beta$ forms of the protein (Bass et al., 1992), and therefore did not fully address this question. The data presented here clearly demonstrate that isolated maize ribosomes are quite resistant to both the pro- and activated $\alpha\beta$ forms of maize RIP, whereas they are readily depurinated by a heterologous RIP from pokeweed.

The insensitivity of maize ribosomes to activated RIP is further demonstrated by the fact that we have expressed both maize pro-RIP and RIP-ΔNIC genes under the control of a constitutive cauliflower mosaic virus 35S promoter in stable transgenic maize callus tissues at levels of approximately 0.01% total protein without any apparent deleterious effect. Similar experiments in tobacco differ: we have recovered many transgenic plants stably expressing the inactive pro-RIP, whereas we did not recover transgenic plants expressing the activated RIP-ΔNIC gene (C. Poirier, A. Morgan, T. Hey, and T. Walsh, unpublished results). In vitro aniline release assays showed that ribosomes from tobacco were insensitive to treatment with pro-RIP at concentrations up to 1.5 μM, but were sensitive to equivalent levels of $\alpha\beta$ RIP (data not shown). Although these in vitro and transgenic experiments may not accurately reflect in vivo conditions during RIP accumulation within endosperm cells, it is apparent that an inactive pro-form of RIP is not essential for the protection of maize ribosomes. Consistent with this conclusion is the observation that nonsecreted RIPs accumulate in wheat and barley endosperms without the need for inactive precursors (Leah et al., 1991; Habuka et al., 1993). If the pro-form of maize RIP is not required for protection of the plant's ribosomes, then the role of the acidic pro-segments of maize RIP may not be restricted to modulation of enzyme activity. For example, they may neutralize the highly basic $\alpha\beta$ RIP (pI = 9.5) to facilitate cytoplasmic accumulation, or their proteolysis during germination may release scarce amino acids such as Met, which is 9 mol % of the pro-segments compared with 2 mol % of the $\alpha\beta$ RIP.

It is becoming clear that the specificity and potency of RIPs from different sources vary (Barbieri et al., 1993). Many have broad activity against prokaryotic and eukaryotic ribosomes (e.g. pokeweed antiviral protein, dianthin, saporin, *Mirabilis* antiviral protein), whereas some have no or limited activity against prokaryotic and plant ribosomes (e.g. gelonin, cereal RIPs). Other factors influence the susceptibility of ribosomes to inactivation by certain RIPs, e.g. the requirement for ATP or other cofactors (Coleman and Roberts, 1981; Sperti et al., 1991; Carnicelli et al., 1992; Brigotti et al., 1993), and these may be linked to the specificity of RIPs. The amount and precise timing of RIP accumulation, particularly in seeds, may also be involved in determining the susceptibility of cellular protein synthesis to the action of a RIP in vivo. A unique RIP, JIP60, has recently been described from barley (Reinbothe et al., 1994). It is induced by methyl jasmonate and is reported to have differential activity against host ribosomes, depending on the stress condition of the plant, although the mechanisms involved in the selectivity have not been elucidated. Appreciation of the details of RIP action will be required to interpret results of experiments involving transgenic expression of RIPs.

Maize pro-RIP is converted to the active form by proteolysis, which removes peptide segments from the N and C termini and also from the center of the polypeptide (Walsh et al., 1991). Several other type-1 RIPs undergo processing at either the N or C termini, although none have been reported to have internal processing and none of the processed regions has been shown to inactivate the RIP (Chow et al., 1990; Benatti et al., 1991; Ho et al., 1991; Kataoka et al., 1991, 1992; Legname et al., 1991). N-terminal processing has generally been associated with presequences that specify translocation into the ER, whereas C-terminal processing has been associated with vacuolar targeting (Benatti et al., 1991; Legname et al., 1991). Maize RIP is cytosolic (DiFonzo et al., 1986) and processing is therefore unlikely to be associated with organellar targeting; rather, it appears to be directly related to RIP activity. To understand the contribution of the processed segments on the ability of maize RIP to inhibit protein synthesis, a series of genetic constructions was made that selectively deleted, either separately or in combination, the N-terminal, C-terminal, or internal processed segments. The deletion mutants were then expressed at high levels in *E. coli*, purified, and tested for RIP activity.

Because some RIPs have significant activity against *E. coli* ribosomes (Habuka et al., 1990; Hartley et al., 1991; Girbes et al., 1993a), heterologous expression can be problematic. In the case of *Mirabilis* antiviral protein (a RIP), reasonable levels of expression were obtained only by using a temperature-sensitive expression system and secretion of the protein via the *ompA* signal sequence (Habuka et al., 1990). Recently, however, successful bacterial overexpression of some type-1 RIPs has been reported (Habuka et al., 1993; Nolan et al., 1993). We have found that all forms of maize RIP can be expressed at very high levels in *E. coli* and so appear to have little or no effect on *E. coli* ribosomes.

The recombinant maize pro-RIP produced in *E. coli* is fully functional, since it can be readily converted by treatment with the nonspecific protease subtilisin Carlsberg into an $\alpha\beta$ RIP form that has activity comparable to that of native $\alpha\beta$ RIP purified from maize kernels. The results of our deletion experiments clearly identify the 25-amino acid insertion as the major inactivating element in the pro-RIP. Removal of the insertion accounts for an increase in activity of at least 5000-fold. In contrast, the removal of the N- and C-terminal segments increases activity only slightly (5- to 10-fold) in the absence of the insertion. This is in contrast to the majority of other zymogen forms of enzymes, particularly proteinases, in which removal of the N-terminal propeptide results in activation (Neurath, 1989). The RIP construction with all three processed regions (RIP-ΔNIC) deleted has an activity close to that of the native $\alpha\beta$ form. The α and β portions of the polypeptide chain are fused in this construction, demonstrating that the polypeptide chain of maize RIP does not have to be cleaved to be active. This

fusion protein (RIP-ΔNIC) is also quite stable to proteolysis and is therefore analogous to typical type-1 RIPs from other plant species.

Alignment of the amino acid sequences of maize RIP and ricin A chain indicates that the maize RIP insertion is at a position equivalent to Thr^{156} of ricin A (Walsh et al., 1991). This places the insertion in a surface loop in ricin A allowing access to processing proteases, and at a position not directly associated with the active site cleft of the enzyme. The loop where the insertion occurs connects helices D and E. Helix E runs through the core of the ricin A molecule, the distal end of which contains Glu^{177} and Arg^{180}, which have been strongly implicated in catalysis (Frankel et al., 1990; Katzin et al., 1991). The presence of the insertion may disrupt the conformation of helix E and therefore the position of the active site residues, such that it renders the enzyme catalytically inert. A mutation in ricin A that is not in the active site region has also been shown to significantly reduce the catalytic activity (Gould et al., 1991). However, the interaction of RIPs with ribosomes may not be limited to active site/ribosomal RNA contacts, and the involvement of ribosomal proteins and/or other translation factors may be critical (Sperti et al., 1991; Ippoliti et al., 1992; Brigotti et al., 1993). The various RIP derivatives that we have prepared will be useful in probing these details.

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